

**ISOLATION AND CHARACTERIZATION OF
ANTIOXIDANT COMPOUNDS FROM PYROLIGNEOUS
ACID OF *RHIZOPHORA APICULATA***

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ACID OF *RHIZOPHORA APICULATA***

by

LOO AI YIN

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LIST OF ABBREVIATIONS

Antioxidative assays

AA	Ascorbic acid
ABTS	2,2'-Azinobis(3-ethylbenzothiazoline 6-sulfonate)
AEAC	Milligrams of ascorbic acid equivalents per gram sample
BDE	Bond dissociation enthalpy
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CPA	Concentrated pyroligneous acid
CPAE	Dichloromethane extract of concentrated pyroligneous acid
CPAE II	Polyphenols extract of CPAE
DPPH	1,1- Diphenylpicrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
FRAP	Ferric reducing antioxidant power
PA	Pyroligneous acid
TR	Trolox

Gas chromatography - mass spectrometry (GC-MS)

SGCC	Silica gel column chromatography
CH ₂ Cl ₂	Dichloromethane
eV	Electron volt
F	Fraction
FID	Flame ionization detector
GC	Gas chromatography
ID	Internal diameter
m/z	Mass/charge

MS	Mass spectrometry
PTLC	Preparative thin layer chromatography
TLC	Thin layer chromatography
t_R	Retention time (min)
v	Volume

Fourier transform- infrared red (FT-IR)

bend	Bending
br	Broad
m	Moderate
s	Strong
str	Stretching
w	Weak

Nuclear magnetic resonance (NMR)

C	Carbon
CDCl ₃	Deuterated chloroform
d	Doublet
dd	Doublet of doublets
H	Proton
m	Multiplet
ppm	Part per million
s	Singlet
t	Triplet

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PEMISAHAN DAN PENCIRIAN SEBATIAN ANTIOKSIDAN DARIPADA ASID PIROLIGNEUS *RHIZOPHORA APICULATA*

ABSTRAK

Aktiviti antioksidatif asid piroligneus (PA) hasil kondensasi wap daripada pembakaran kayu *Rhizophora apiculata* semasa penghasilan arang kayu telah dikaji. PA dipekatkan kepada 10:1 mengikut isipadu pada 80 °C dengan menggunakan alat pemeruap berputar bertekanan rendah bagi menghasilkan asid piroligneus pekat (CPA). CPA ini kemudiannya diekstrak dengan diklorometana bagi menghasilkan ekstrak diklorometana asid piroligneus pekat (CPAE). Jumlah kandungan fenolik, aktiviti pemerangkapan radikal bebas DPPH, kuasa penurunan ion ferrik dan ion molibdenum (VI) bagi PA, CPA dan CPAE telah dikaji. Keputusan menunjukkan CPAE memiliki kuasa antioksidatif yang kuat dan komponen fenolik merupakan penyumbang utama kepada kuasa antioksidatif asid piroligneus, *Rhizophora apiculata*. Fraksi fenolik (CPAE II) telah diekstrak daripada CPAE dengan menggunakan pengekstrakan asid-bes dan pengekstrakan pelarut. Komposisi kualitatif dan kuantitatif CPAE II dikaji dengan kaedah spektroskopi gas (GC) dan kaedah kromatografi gas-spektrometri (GC-MS). Sebanyak 52 komponen utama yang mewakili 95.47 % daripada jumlah kandungan CPAE II telah berjaya dipastikan identitinya. Analisa kuantitatif menunjukkan CPAE II didominasi oleh syringol (49.34 %), maltol (12.45 %), katekol (5.37 %) dan 2-hidroksi-3-metil-2-siklopentenon (4.69 %). Seterusnya, CPAE II diperingkatkan dengan kaedah kromatografi plat nipis (TLC) dan kaedah turus kromatografi gel silika (SGCC) bagi menghasilkan empat pecahan utama (F1-F4). Keempat-empat pecahan tersebut kemudiannya diperingkatkan lagi dengan pelbagai kaedah kromatografi seperti SGCC dan kromatografi plat nipis penyediaan (PTLC). Lapan komponen telah diasingkan dan dikenalpasti termasuk katekol, 3-

metoksikatekol, vanillin, eugenol, syringol, *p*-kresol, 2-hidroksi-3-metil-2-siklopentenon dan maltol. Struktur komponen dikenalpasti secara kaedah spektroskopi dan kromatografi termasuk FT-IR, GC-MS, ¹H-NMR dan ¹³C-NMR. Bagi memperoleh keputusan muktamad, kaedah penyuntikan serentak GC dengan komponen piawai telah dilakukan. Potensi antioksidatif kelapan-lapan komponen telah dikaji dengan lima kaedah klasik termasuklah aktiviti pemerangkapan radikal bebas DPPH, kuasa penurunan ion ferrik dan ion molibdenum (VI), aktiviti pemerangkapan radikal kation ABTS dan kuasa pengkelatan ion ferrus. Komponen yang berlainan memperlihatkan kuasa antioksidatif yang berlainan, bergantung kepada struktur molekul masing-masing. Katekol menunjukkan kuasa pemerangkapan radikal DPPH dan kuasa penurunan ion ferrik yang paling kuat, sementara 3-metoksikatekol menunjukkan kuasa pemerangkapan radikal kation ABTS dan kuasa penurunan ion molibdenum (VI) yang paling tinggi. Ini disebabkan oleh kehadiran dua kumpulan hidroksi yang bersebelahan dalam struktur kedua-dua komponen ini. Maltol merupakan agen pengkelatan yang paling kuat dan sifat ini dikaitkan dengan kehadiran satu kumpulan hidroksi pada atom karbon ketiga disertai dengan satu kumpulan keto pada atom karbon keempat dalam strukturnya. Kuasa antioksidatif asid piroligneus, *Rhizophora apiculata* dan kelapan-lapan komponen antioksidatif di dalamnya telah berjaya dipisah dan dicirikan buat pertama kalinya.

ISOLATION AND CHARACTERIZATION OF ANTIOXIDANT COMPOUNDS FROM PYROLIGNEOUS ACID OF *RHIZOPHORA APICULATA*

ABSTRACT

The antioxidative activity of the pyroligneous acid (PA), a vapours condensate of the production of charcoal from *Rhizophora apiculata* mangrove was evaluated. The PA was concentrated to a 10:1 volume ratio using a reduced pressure rotary evaporator at 80 °C to produce concentrated pyroligneous acid (CPA). Dichloromethane extraction of CPA successfully yield another extract, namely the concentrated pyroligneous acid extract (CPAE). Total phenolic content, DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP) and molybdenum (VI) ion reducing activity of PA, CPA and CPAE were evaluated. Results showed that CPAE was amongst the strongest antioxidant and that polyphenols were the major components contributed to its antioxidative activity. The polyphenols fraction (CPAE II) was isolated from CPAE by simultaneous acid-base and solvent extraction methods. Its qualitative and quantitative composition was accomplished with gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS), respectively. A total of 52 compounds were successfully identified and quantified, representing 95.47 % of the total polyphenols content of CPAE II. Quantification analysis showed that CPAE II was dominated by syringol (49.34 %), maltol (12.45 %), catechol (5.37 %) and 2-hydroxy-3-methyl-2-cyclopentenone (4.69 %). CPAE II was then fractionated into four fractions (F1 - F4) by means of thin layer chromatography (TLC) and silica gel column chromatography (SGCC). F1-F4 were then subjected to further purification by means of various chromatographic techniques including SGCC and preparative thin layer chromatography (PTLC). The separations resulted in the isolation of eight compounds, catechol, 3-methoxycatechol, vanillin, eugenol, syringol, *p*-cresol, 2-hydroxy-3-methyl-

2-cyclopentenone and maltol. Their structures were identified by GC-MS, ¹H-NMR, ¹³C-NMR spectral analyses, and confirmed by GC co-injection with authentic standards. The antioxidative activities of the isolated compounds were evaluated by DPPH radical scavenging, ABTS radical cation scavenging, phosphomolybdenum, ferric reducing antioxidant power and ferrous chelating assays. Different compounds differed significantly in their antioxidative activities, as well as their structure-antioxidative capacity relationships in the five assays tested. Catechol and 3-methoxycatechol showed the greatest antioxidative activity analyzed by the DPPH radical scavenging and FRAP assay, phosphomolybdenum and ABTS radical cation scavenging assay, respectively. The strong antioxidative activities shown by these two compounds were attributed to the presence of the di-ortho phenolic structure. Maltol appeared to be the strongest ferrous ion chelator due to the presence of a 3-hydroxyl group in conjunction with a C4 keto group in the structure. The antioxidative activities of *Rhizophora apiculata* pyroligneous acid and the isolation and characterization of the eight antioxidants were the first ever successfully evaluated.

CHAPTER ONE

INTRODUCTION

1.1 Definition of pyroligneous acid

Pyroligneous acid is the name of the crude condensate produced from the distillation of smoke generated in the process of making charcoal. The word “pyroligneous” comes from “pyrolysis” involving wood and “lignin” as one of the components in wood. Pyrolysis is a thermal process that rapidly heats biomass (such as wood) in an oxygen-free environment to a carefully controlled temperature, and then very quickly cools the volatile products formed during the reaction. The main objective of wood pyrolysis is to produce charcoal, liquid and gaseous products. This process is also the source of many basic organic chemicals, such as acetic acid, aldehydes, ketones, methanol and acetone (Demirbaş & Güllü, 1998).

In the common practice of charcoal production, by heating the wood in the absence of air, vapours and gases escape into the atmosphere as smoke. The smoke produced can be recovered by passing it through a stainless steel pipe for condensation to yield the pyroligneous acid, while the non-condensable wood gas which can be used as fuel (Güllü & Demirbaş, 1998).

Thus, more specifically, pyroligneous acid can be defined as the by-product with a complex mixture of compounds derived from the chemical break-down of the components in wood (cellulose, hemicellulose and lignin) through the condensation of vapours generated during the pyrolysis of wood in an oxygen-free environment.

At present, there are many synonyms given to pyroligneous acid such as wood vinegar, liquid smoke, wood smoke, wood liquids, wood oil, wood distillate, wood tar, smoke flavouring, smoke condensate, pyrolysis oil, bio-crude-oil, bio-oil, bio-fuel-oil, and pyrolysis liquids. In Japan, it is more commonly known as “mokusaku-eki” (Adjaye, Sharma & Bakhshi, 1992; Ikegami, Toshikazu & Yuichi, 1998; Oasmaa & Peacocke, 2001). Such pyroligneous acid is extracted as a by-product in the manufacture of charcoal, and it is usually discarded due to insufficient applications.

1.2 Physical properties of pyroligneous acid

Typically, pyroligneous acid appears to be a reddish brown liquid which resembles to the pleasing hue of black tea, beer or wine. Pyroligneous acid can range in colour from dark green or dark red through black, depending upon the feedstock and process used to manufacture the product. The colour may also vary depending on the type of wood used and on climatic condition. The pH value of pyroligneous acid is low, ranging from 2 - 3, due to its high amounts (8 - 10 wt %) of volatile acids, mainly acetic acid and formic acid (Sipila *et al.*, 1998). These acids are the main components responsible for the mild corrosive properties of pyroligneous acid (Aubin & Roy, 1980).

Typically, pyroligneous acid is a free flowing liquid. It consists of two phases, an aqueous phase and a non-aqueous phase. The former is composed of a wide variety of organo-oxygen compounds of low molecular weight such as acetic acid, acetone and methanol. The latter, also known as wood tar or bio-oil (Adjaye, Sharma & Bakhshi, 1992) contains insoluble organics (mainly aromatics) of high molecular weight such as phenols and derivatives (Maschio, Koufopoulos & Lucchesi, 1992).

Pyroligneous acid has been described to have a pleasant aroma. Its aroma, however, may differ depending on the nature of the material involved in its preparation (Guillén & Manzanos, 2005). Pyroligneous acid which is produced from the same species of wood and the same smoke generation condition may create distinctive flavour and composition (Guillén, Manzanos & Zabala, 1995). In general, the flavour compounds such as phenol derivatives, furan and pyran derivatives, aldehydes and ketones, esters and nitrogenated derivatives are important contributors to the global smoke aroma. The physical properties of pyroligneous acid are summarized in Table 1.1.

Table 1.1 Typical physical properties of pyroligneous acid (Oasmaa & Peacocke, 2001).

Physical Properties	Values
Appearance	Reddish brown liquid
Acidity (pH)	2 – 3
Viscosity (cSt)	20-100 at 40 °C
Flash point (°C)	44 – 46
Boiling curve	Starts boiling below 100 °C
Auto ignition temperature (°C)	Approximately above 500
Specific gravity	1.070 - 1.090 at 25 °C
Vapour pressure	Similar to water
Odour	Pleasant-smoky aroma

1.3 Chemical composition of pyroligneous acid

Chemically, pyroligneous acid consists of water, water-soluble organic compounds and water-insoluble organic compounds (Oasmaa & Peacocke, 2001).

As shown in Table 1.2, pyroligneous acid contains a high number of compounds arising from wood carbohydrate thermal degradation such as aldehydes, ketones, diketones, esters, alcohols, acids, furan and pyran derivatives. In addition, it also contains a significant number of components arising from lignin thermal degradation

(Fengel & Wegener, 1983; Shafizadeh, 1984) such as phenol, guaicol, syringol, pyrocatechol and their derivatives, as well as a trace amount of some other components (Guillén & Manzanos, 1999; 2002; Guillén, Manzanos & Ibargoitia, 2001).

Table 1.2 Compounds detected in pyrolygneous acid (Guillén & Manzanos, 1999; 2002; Guillén, Manzanos & Ibargoitia, 2001).

Families	Compounds
Guaicol and derivatives	2-Methoxyphenol (guaicol)
	4-Methyl -2-methoxyphenol
	1-(3-Hydroxy-2-methoxyphenyl)-ethanone
	4-(2-Propenyl)-2-methoxyphenol (eugenol)
	4-Hydroxy-3-methoxybenzaldehyde (vanillin)
	4-Hydroxy-3-methoxybenzoic acid (vanillin acid)
	1-(4-Hydroxy-3-methoxyphenyl)-2-propanone
	1-(4-Hydroxy-3-methoxyphenyl)-ethanal
Syringol and derivatives	2,6-Dimethoxyphenol (syringol)
	3,4-Dimethoxyphenol
	4-Methyl-2,6-dimethoxyphenol
	4-Ethyl-2,6-dimethoxyphenol
	4-Vinyl-2,6-dimethoxyphenol
	4-Propyl-2,6-dimethoxyphenol (4-propylsyringol)
	4-Hydroxy-3,5-dimethoxybenzaldehyde
	1-(4-Hydroxy-3,5-dimethoxyphenyl)-ethanone
	1-(4-Hydroxy-3,5-dimethoxyphenyl)-2-propanone
Pyrocatechol and derivatives	1,2-Benzenediol (catechol)
	1,4-Benzenediol (hydroquinone)
	3-Methoxy-1,2-benzenediol
	3-Methyl-1,2-benzenediol
	4-Methyl-1,2-benzenediol
	4-Ethylbenzenediol
Carbohydrate derivatives	1,6-Anhydro- α -D-galactofuranose
	1,6-Anhydro- α -D-galactopyranose
	1,6-Anhydro- β -D-mannopyranose
Terpenic compounds	Camphor
	Terpinen-4-ol
	5-Hydroxy-1,8-cineole
	2H-1-Benzopyran-2-one

Table 1.2 Continued

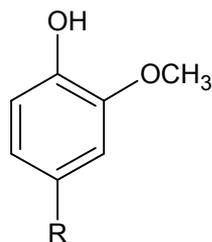
Families	Compounds
Nitrogenated compounds	2-Methylpyridine
	3-Methylpyridine
	3-Methoxypyridine
	1,3-Dimethyl-1H-pyrazole
	2-Ethyl-6-methylpyridine
	2-Methyl-3-pyrinidol

Phenol and its derivatives have been considered as the primary contributors to smoke aroma, while furan and pyran derivatives were commonly known in softening the heavy aroma associated with phenolic compounds (Kim, Kurata & Fujimaki, 1974; Radecki *et al.*, 1977; Kjällstrand & Petersson, 2001a). Phenolic derivatives have been described to have pungent cresolic, sweet, smoky and burnt odour (Kim, Kurata & Fujimaki, 1974; Baltés *et al.*, 1981; Toth & Potthast, 1984). Carbonyl derivatives have been attributed to caramel or burnt sugar notes (Fiddler, Wasserman & Doerr, 1970). Examples of some of the flavour compounds in pyrolygneous acid are listed in Table 1.3.

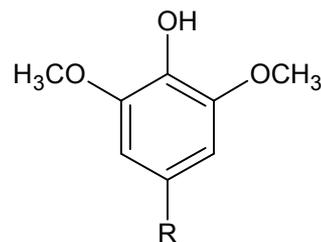
Table 1.3 Examples of the flavour compounds in pyrolygneous acid (Kim, Kurata & Fujimaki, 1974; Toth & Pottast, 1984; Buckingham, 1994; Guillén & Manzanos, 1999; Murwanashyaka, Pakdel & Roy, 2001).

Compounds	Odours
3-Methyl-2-cyclopenten-1-one	Sweet, woody
3-Hydroxy-2-methyl-4H-pyran-4-one	Caramel, butterscotch
3-Methyl-2(5H)-furanone	Sweet, like burnt caramel
2-Methoxyphenol (guaicol)	Sweet, smoky, somewhat pungent
2,6-Dimethoxyphenol (syringol)	Woody, smoky
3-Methyl-1,2-cyclopentanedione	Brandy or caramel, smoked ham, cloves.

In fact, phenol and its derivatives in pyrolygneous acid are valuable chemicals having a wide range of applications in industry, home and pharmaceutical products. The most widely known and studied phenols in pyrolygneous acid are methoxyphenols bearing the guaiacyl (1.1) and syringyl (1.2) structure (Kjällstrand & Petersson, 2001b).



(1.1)



(1.2)

1.4 Production of pyrolygneous acid

1.4.1 Wood pyrolysis

Pyrolysis is the thermal degradation of carbonaceous material in an oxygen-free environment to a carefully controlled temperature, and then very quickly cools the volatile products formed during the reaction (Sinha *et al.*, 2000). Wood is thermally degradable and can be converted into solid residue (charcoal), liquid, and gaseous products by pyrolysis (Güllü & Demirbaş, 2001; White & Dietenberger, 2001).

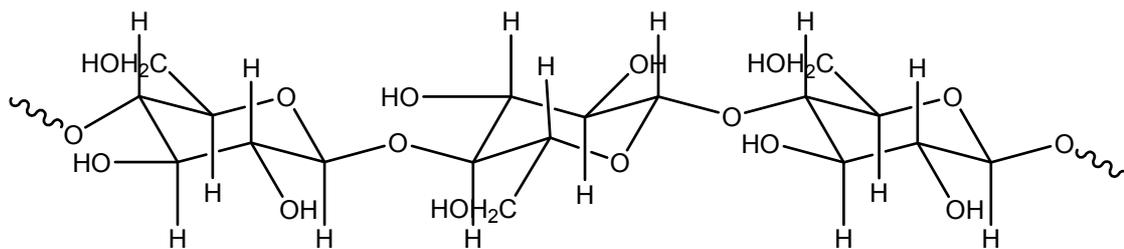
For a high yield of charcoal, a low temperature and low heating rate process should be chosen (Güllü & Demirbaş, 2001). In addition, moisture content, wood size and wood species also play an important role in wood pyrolysis. Woods for pyrolysis purposes should be as dry as possible as any moisture in wood will extend the pyrolysis process,

and decrease the percentage (%) yield (Zaror & Pyle, 1984), thus increase the cost of production.

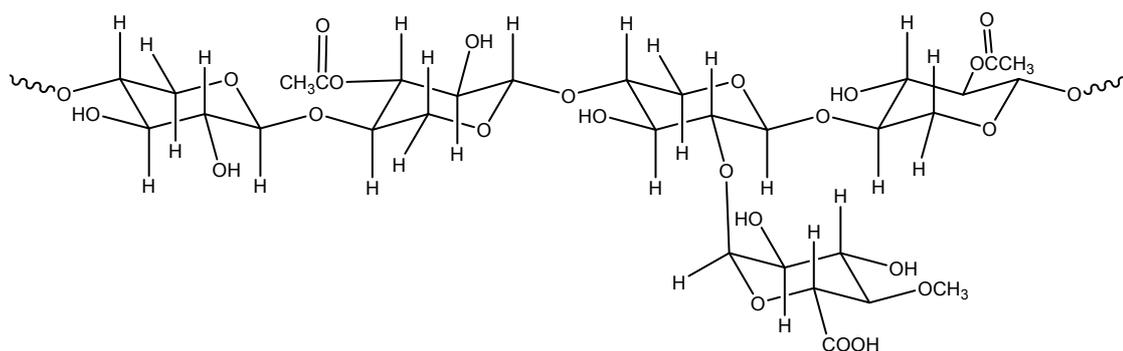
Wood consists of three main components, cellulose (1.3), hemicellulose (1.4) and lignin (1.5). There are some variation in the relative abundance of these constituents in different species of wood but as rough guideline, cellulose (1.3) is taken to be approximately 50% by dry weight of wood and the other two components contribute approximately 25% each to the dry weight of wood (LeVan, 1989; Guillén & Manzanos, 1999; Sinha *et al.*, 2000; Güllü & Demirbaş, 2001; Simko, 2005; Stolyhwo & Sikorski, 2005).

Cellulose (1.3) is a glucan polymer consisting of linear chains of β (1,4) D-glucopyranose units (Serio, Wojtowicz & Charpenay, 1995). It has an average molecular weight of 100,000 Da. Aggregation of these linear chains within the microfibrils provides a crystalline structure that is highly inert and inaccessible to chemical reagents (Sinha *et al.*, 2000).

Hemicellulose (1.4) is a complex mixture of polysaccharides. It is mainly composed of glucose, mannose, galactose, xylose, arabinose and galacturonic acid residues (Serio, Wojtowicz & Charpenay, 1995). Generally, it has a much lower molecular weight than that of cellulose (1.3), and is amorphous in structure (Sinha *et al.*, 2000). Hemicellulose (1.4) is thermally more sensitive and decomposes during the earlier stages of pyrolysis (LeVan, 1989; Morf, 2001).

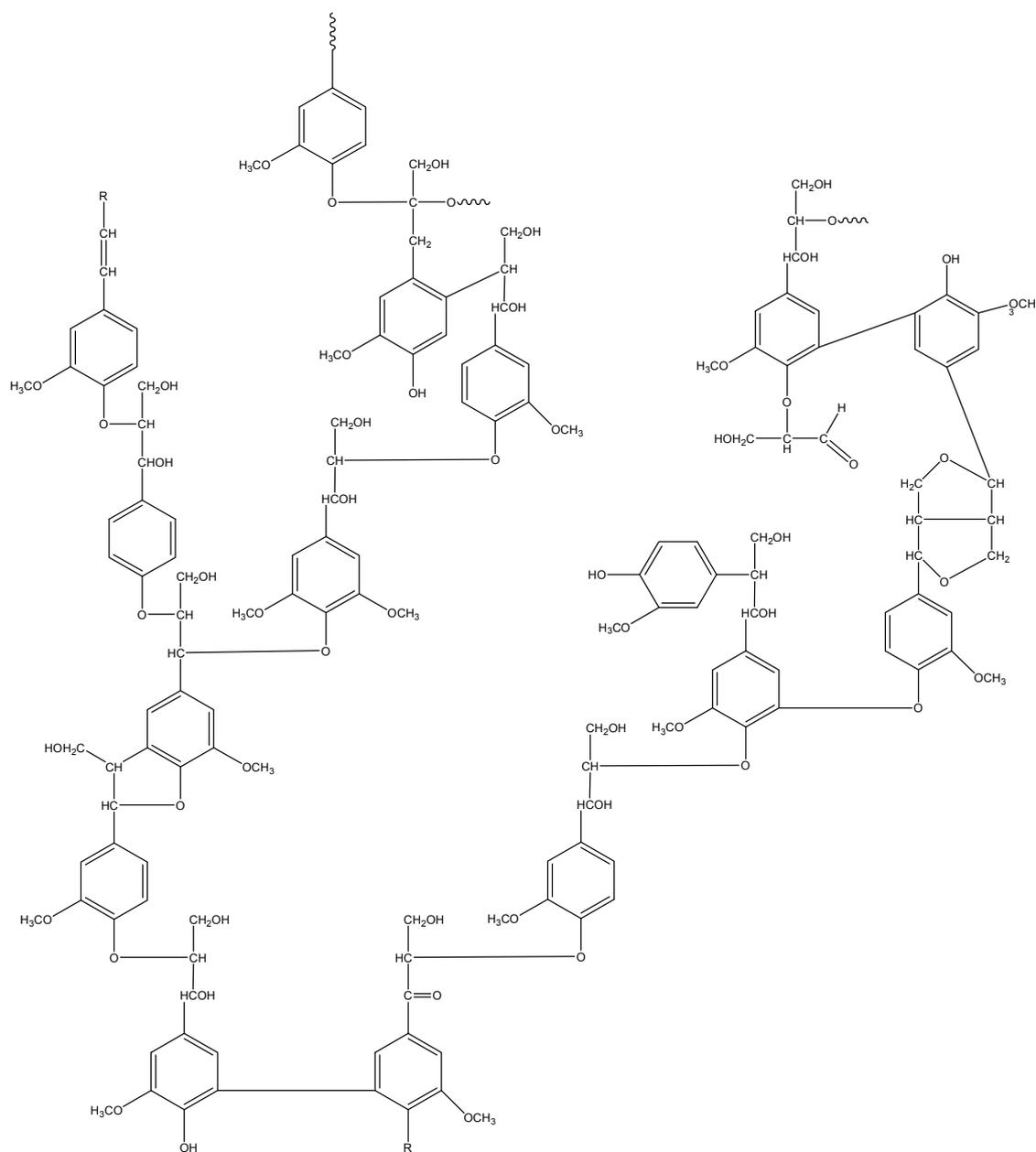


(1.3)



(1.4)

Lignin (1.5) is a complex, naturally occurring phenolic phenylpropanoid polymer (Serio, Wojtowicz & Charpenay, 1995). Lignin (1.5) is the last component to pyrolyse in wood (Güllü & Demirbaş, 2001; Morf, 2001). It is amorphous in nature and can be processed to yield a range of aromatic compounds (Sinha *et al.*, 2000).



(1.5)

Primary wood pyrolysis involves the simultaneous decomposition of cellulose (1.3), hemicellulose (1.4) and lignin (1.5) in wood into charcoal and volatiles products (Sinha *et al.*, 2000). The secondary pyrolysis reaction which involves the decomposition of products of primary reactions will be initiated when these hot volatiles products come into contact with unpyrolysed sawdust (Sinha *et al.*, 2000). The overall process of

pyrolysis of wood can be divided into four main stages based on the increasing temperature of pyrolysis in the charcoal kiln (Levan, 1989; Güllü & Demirbaş, 2001; White & Dietenberger, 2001; Simko, 2005; Sinha *et al.*, 2000; Stolyhwo & Sikorski, 2005).

At zone A (≤ 200 °C), wood becomes dehydrated and water vapour and other non-combustible gases and liquids including carbon dioxide, formic acid, acetic acid and water were generated. When the temperature increases and reaches zone B (200 - 280 °C), hemicellulose (1.4) starts to decompose yielding volatile products such as carbon dioxide, carbon monoxide, water, acetic acid and high-boiling tar. Cellulose (1.3) remains unpyrolysed at this temperature, however as the temperature increases, depolymerization of cellulose (1.3) occur to yield carbonyl and carboxyl groups of compounds. This is an endothermic reaction and the wood is becoming charred. When the temperature reaches 280 - 500 °C (zone C), decomposition of cellulose (1.3) begins and temperature reaches maximum at 320°C approximately. At 320 - 500 °C, decomposition of lignin (1.5) is started. The aliphatic side chains in the lignin (1.5) start splitting off from the aromatic ring and eventually, the carbon-carbon linkage between lignin (1.5) structural units is cleaved to yield the phenolic compounds. This is an exothermic reaction and continues until only the carbonized residue called charcoal remains. In addition, carbon monoxide, methane, formaldehyde, formic acid, acetic acid, methanol and hydrogen gas are also being liberated. If the temperature continues to rise above 500 °C, the wood is said to be in zone D. This is where the secondary pyrolysis reactions come into account. The remaining wood residues undergo further degradation by being oxidized to carbon dioxide, carbon monoxide, and water. Charcoal is being produced at zone C and D. Table 1.4 summarizes the processes in the pyrolysis of wood.

Table 1.4 Four major stages of wood pyrolysis (LeVan, 1989; Güllü & Demirbas, 2001; White & Dietenberger, 2001; Simko, 2005; Stolyhwo & Sikorski, 2005).

Temperature	Degraded Wood Components	Products
Zone A (≤ 200 °C)	Water moisture present in wood	Carbon dioxide, formic acid, acetic acid and water.
Zone B (200-280 °C)	Hemicellulose (1.4)	Carbon dioxide, water, carbon monoxide, acetic acid, high boiling tar some carbonyl and carboxyl compounds.
Zone C (280-500 °C)	Cellulose (1.3) at 200-320 °C Lignin (1.5) at 320-500 °C	Carbon monoxide, hydrogen, methanol, acetic acid, formic acid, formaldehyde, methane carbonyl, charcoal and phenolic compounds.
Zone D (< 500 °C)	Wood residue, primary products	Carbon dioxide, carbon monoxide, water and charcoal.

1.4.2 Production of charcoal and pyroligneous acid in Malaysia

1.4.2.1 Mangrove *Rhizophoraceae* forests in Malaysia

Mangroves are trees and shrubs that grow in saline coastal habitats in the tropics and subtropics. The word is used in at least three senses, (1) most broadly to refer to the habitat and entire plant assemblage or mangal, for which the terms mangrove swamp and mangrove forest are also used, (2) to refer to all trees and large shrubs in the mangal, and (3) narrowly to refer to the mangrove family of plants, the *Rhizophoraceae*, or even more specifically just to mangrove trees of the genus *Rhizophora* (Hogarth, 1999).

Mangroves constitute a rich, diverse and complex ecosystem which is generally a productive resource base. At present, Malaysia has 64,000 ha of mangrove forest remaining, of which 446,000 ha (70%) is gazetted as mangrove forest reserves that are managed for sustainable forestry production as part of the Permanent Forest Estate. The other 30% is state land mangrove, which come under the jurisdiction of the respective state governments. About 57% of Malaysia's mangrove forests are found in Sabah, 26% in Sarawak and 17% in Peninsula Malaysia (Clough, 1993).

In Peninsula Malaysia, the state of Perak has the greatest number of mangrove reserves of which 19 reserves form Matang mangroves forest in Kuala Sepetang, Taiping. The Matang Mangrove Forest Reserve (4° 50'N, 100° 35' E) is situated in the State of Perak, on the northwest coast of peninsular Malaysia (Fig.1.1). Matang mangroves in Malaysia are among the best managed forests in the world (Harron, 1981). It stretches for 51 km along the Perak coastline between Kuala Gula and Bagan Panchor, and measures 13 km at its broadest point. It has been acknowledged as the most well managed system in the world. The mangrove species in this forest are predominantly *Rhizophora apiculata* (Fig.1.2a) and *Rhizophora mucronata* (Fig.1.2b) (Koh, The Star, 2004). Current management plans call for a 30-year rotation with two thinnings between 10-15 year and 20-25 year. After 30 years, the mangrove trees will be harvested for charcoal, firewood or poles (Alongi, 1998).



Fig. 1.1 Location of Matang Mangrove Forest at Kuala Sepetang, Perak, Malaysia.

1.4.2.2 *Rhizophora*

Rhizophora is a genus of tropical trees. The most notable species is the Red mangrove (*Rhizophora mangle*) but other species are known, including *Rhizophora apiculata* and *Rhizophora stylosa*. All *Rhizophora* species have arching stilt roots that emerge from the trunk (Fig.1.2 a, b). These roots help to support the tree in soft mud and also help it to breathe. *Rhizophora* species generally live in inter-tidal areas which are inundated daily by the ocean. They exhibit a number of adaptations to this environment, including stilt-roots that elevate the plants above the water and allow them to respire oxygen even while their lower roots are submerged, and a cytological molecular “pump” mechanism that allows them to remove excess salts from their cells.

1.4.2.3 *Rhizophora apiculata* blume

Rhizophora apiculata blume is one of the major species of *Rhizophora* genus. This is the most preferred species of mangrove in the production of charcoal in Matang mangrove forest as *Rhizophora apiculata* species produces higher quality charcoal than any other species of mangroves and it also does not produce a lot of ashes (Lawrence & Samuel, 1984).



Fig. 1.2 *Rhizophora apiculata* mangrove (a) and *Rhizophora mucronata* mangrove (b).

1.4.2.4 Charcoal and pyroligneous acid production in Kuala Sepetang

In Malaysia, Matang mangrove is known as the largest producer of mangrove charcoal with about 12,000 tonnes exported in the year 2000 (Lim, The Star, 2004). Traditionally, the charcoal is produced on a commercial scale in large (100-200 m³), igloo-shaped masonry-kilns (Fig.1.3a) built near a channel at the edge of the forest (Fig.1.3b).

The process of charcoal making in Matang begins from the cutting of the *Rhizophora apiculata* woods into 1.6 m logs (Fig.1.4a). Followed by the debarking process where the barks with virtually no solid are disposed off (Fig.1.4b, c). Barks have to be

removed to avoid of having high moisture content. This, in turns, will generate large amount of smoke. The debarked wood were then packed closely and stacked in 2 logs high into the kiln (Fig.1.4d). Each charcoal kiln can accommodate approximately 40 tonnes of woods for every carbonization process. A total of 10.5 tonnes of shiny black charcoal will be produced in a single burn (Lim, The Star, 2004).

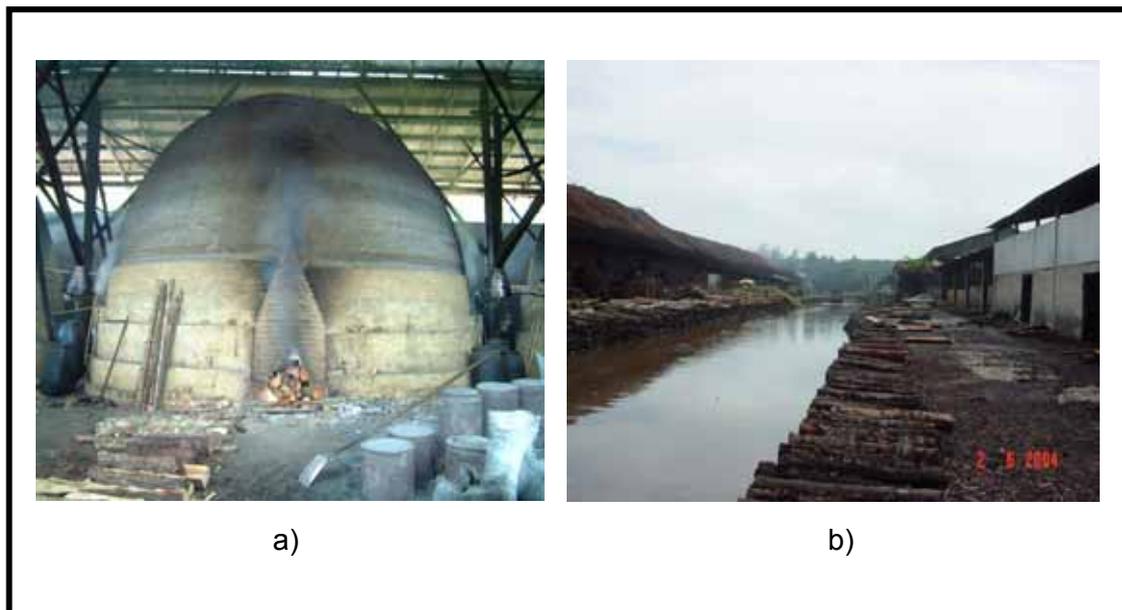


Fig. 1.3 The igloo-shaped charcoal kiln (a) and charcoal factory sites in Kuala Sepetang (b).

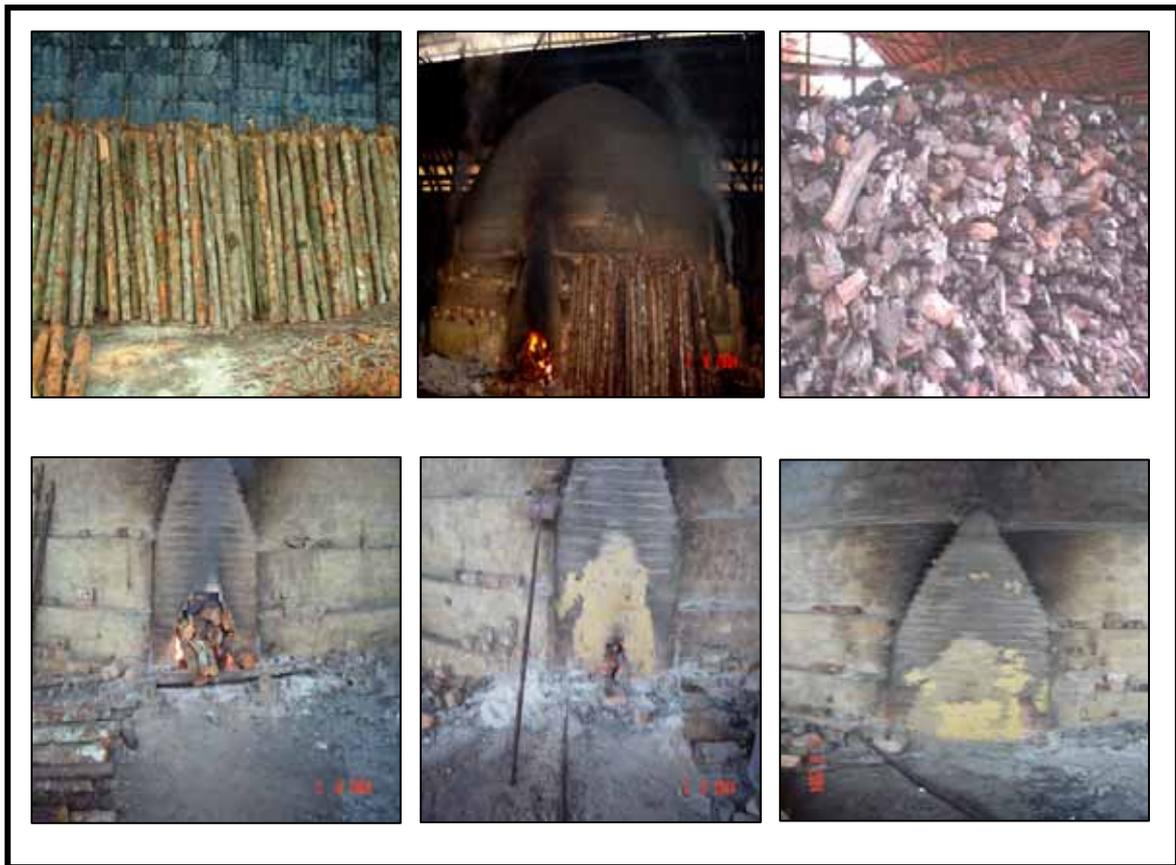


Fig. 1.4 Preparation prior to the charcoal making process at Kuala Sepetang charcoal village. The 1.6 m log of *Rhizophora apiculata* woods (a), the wood debarking procedure (b), the debarked woods (c) packing of the debarked woods into the charcoal kiln (d).

In general, charcoal manufacturing in Kuala Sepetang, Perak involves three major stages (Table 1.5) (Lim, The Star, 2004; Rusli, 2006). The first stage is characterized by the burning of woods for 8-10 days at 100-120°C. At this stage, the moisture and water content in the wood are effectively removed. The next step (Stage II) is characterized by a higher temperature (250°C). At this stage, the entrance of the kiln has to be partially sealed preventing of complete combustion of woods. This stage takes about 12-14 days. The smoke emerging from the orifices of the kiln at 50 - 70°C is collected through stainless steel cone (Fig.1.6a), and is then condensed through air to yield pyroligneous acid. The pyroligneous acid collected has a temperature of 36 - 38°C. During the final stage (Stage III), the woods are left to cool inside the kiln, for 8-9 days approximately. The entrance of the kiln is completely sealed with clay at this stage. The whole process of charcoal manufacturing takes about 28 – 30 days.

Table 1.5 The process of charcoal making in Kuala Sepetang (Lim, The Star, 2004).

Stage	Process	Duration
I	<ol style="list-style-type: none"> 1. The starts of the baking process by lighting firewood at the entrance of the kiln. 2. The temperature in the kiln is 100-120°C. 3. The moisture and water contents in the wood are removed. 	8-10 days
II	<ol style="list-style-type: none"> 1. The opening for the firewood of previous stage is sealed to an even small opening. This is to prevent air from entering the kiln as air will cause a complete combustion of the wood into ashes. 2. The temperature in the kiln is 250°C. 3. Smoke coming out (50-70°C) from the orifices of the kiln is collected by cone-shaped steel and condensed through air. 4. Pyroligneous acid is the result of the condensation of the smoke. 	12-14 days
III	<ol style="list-style-type: none"> 1. The opening of the kiln is totally sealed up, again to prevent complete combustion. 2. The carbonized woods are left to cool in the kiln. 3. Charcoal is the final product at this stage. 	8-9 days



Stage I

Stage II

Stage III

Fig. 1.5 Three major stages in charcoal making process. A small opening was allowed at the entrance of the kiln to accommodate fire woods (I); the entrance had to be partially sealed to prevent complete combustion of woods in the kiln (II); during the final stage, the entrance was totally sealed with clay. This was the cooling process of the carbonized woods. The products obtained were the shiny black charcoals.



Fig. 1.6 The stainless steel pipe for collection of the condensed smoke coming out from the orifice of the charcoal kiln. The reddish brown liquid or pyroligneous acid obtained during the second stage as the result of the condensation of wood smoke (a). The product, after elimination of pyroligneous acid was a thicker, greenish black liquid (b).

1.5 Uses of pyroligneous acid

Pyroligneous acid has been traditionally used as sterilizing agent, deodorizer, fertilizer, antimicrobial and growth promoting agent. It has a wide range of applications in many fields such as industry, agriculture, medicinal and home uses.

1.5.1 Industrial uses

Pyroligneous acid has been used as a source of smoke flavours (Mohan, Pittman & Steele, 2006). Smoke flavours are considered to be Generally Regarded As Safe (GRAS), so they can be used in foods as an additional barrier to prevent microbial growth at levels which comply with good manufacturing practice (Holley & Patel, 2005).

Organisms which play an important role in the spoilage of foods, and bacteria of public health significant, are effectively inhibited by some wood smoke (Sunen, 1998). It was reported that the antimicrobial activity of pyroligneous acid is attributed to the presence of compounds like phenolic compounds, carbonyls and organic acids (Vitt, Himelbloom & Crapo, 2001). Among them, the phenolic compounds probably represent the most important one both from the qualitative and quantitative point of view. The phenolic compounds, and in particular the methoxyphenols, have been considered as the major contributors to smoke aroma and are responsible for the antimicrobial and antioxidant effects in smoked foods (Estrada-Munoz, Boyle & Marsden, 1998; Faith, Yousef & Luchansky, 1992; Guillen & Ibargoitia, 1998; Kjällstrand & Petersson, 2001a; Kjällstrand & Petersson, 2001b; Sunen, Aristimuno & Fernandez-Galian, 2003). Phenolic compounds also contribute to sensory properties of foods, particularly colour and astringency (Garote *et al.*, 2004).

In addition, smoking affects the organoleptic properties of food by imparting a characteristic flavour and taste, and by modifying the texture and the colour (Martinez *et al.*, 2007; Toth & Potthast, 1984). It was reported that mayonnaises with added pyroligneous acid had a higher intensity of whiteness and colour tone than mayonnaises without pyroligneous acid (Wendin, Ellekjaer & Solheim, 1999). Acetic acid, one of the major constituents of pyroligneous acid helps to reduce noxious odours such as ammonia, sulphur and others from decomposing compounds. It has been used as a deodorizer in industries such as paper processing, wastewater processing, organic synthetic processing, refining petroleum processing, oil and fat processing, and to lessen bad odour at high temperature furnace's slag processing device.

1.5.2 Agricultural uses

Pyroligneous acid has long been used as a natural organic pesticide. Pyroligneous acid can reduce the growth of phytopathogenic fungi, such as *Fusarium*, *Pythium* and *Rhizoctonia*; thus, it helps to increase growth of plant roots (Tsuzuki *et al.*, 1989; Kadota *et al.*, 2002). These effects may be due to recovery from transplantation injury, especially to roots, hastening of root growth or prevention of disease injury (Kadota & Niimi, 2004).

The mixture of pyroligneous acid and charcoal has significant beneficial effects in improving growth and yield for field cultivated crops (Tsuzuki *et al.*, 1989; Uddin *et al.*, 1995a; b; Du *et al.*, 1997; Kadota & Niimi, 2004). By adding charcoal to soil, it will result in an increased in plant growth and crop yields (Glaser, Lehmann & Zech, 2002). This is due to the fact that charcoal is porous, in that the permeability of soil by water was increased (Konno & Nishikawa, 1993).

1.5.3 Medicinal and home uses

Pyroligneous acid has been used as an antiseptic, anti-inflammatory and anti-allergic agent. It is also added in laundry or water to facilitate dirt removal, alleviate damage caused by residual chemical detergents and maintaining antibacterial effect in clothing.

With its detoxifying properties, pyroligneous acid is one of the main ingredients used in wood vinegar patches, a health product applied on the human body or soles of the feet to improve blood circulation and promote detoxification function of the body.

1.5.4 Others uses

Nowadays, the rise of oil prices and the growing environmental concern over combustion of fossil fuels such as air pollution, acid rain and greenhouse effect have made bio-fuels more demanding and pyroligneous acid will be a good alternative. When heated to near the boiling point of water, it is possible to be ignited. However, the problems associated to pyroligneous acid as a fuel oil are its high water content which causes higher ignition temperature, while its low pH value (pH 2-3) tends to cause corrosions (Oasmaa & Peacocke, 2001).

1.6 Previous studies on *Rhizophora apiculata* blume

It has been reported that the heartwood of *Rhizophora apiculata* was active against fungi, bacteria and boll weevils. Five long chain aliphatic alcohols, 11 long chain aliphatic saturated carboxylic acids, three steroids, 2,6-dimethoxy-*p*-benzoquinone, syringaldehyde and sitosteryl 3-glucoside have also been isolated from the heartwood of *Rhizophora apiculata* (Kokpol *et al.*, 1993).

The polysaccharide extracted from the leaf of *Rhizophora apiculata* was assessed in cell culture systems, for its activity against human immuno-deficiency viruses. The study indicated that a polysaccharide extracted from *Rhizophora apiculata* has anti-HIV activity *in vitro*, apparently due to interference with the adsorption of virus particles to positive human cells (Premanathan *et al.*, 1999).

Also, the fresh bark of *Rhizophora apiculata* collected from the Pitchavaram mangrove forest (latitude 11°27'N; longitude 79°47'E), Tamil Nadu, India was tested for its free

radical scavenging activity and protective role against mitochondrial dysfunction in naphthalene stressed rats. The results showed that *Rhizophora apiculata* bark extract attenuated mitochondrial dysfunction in naphthalene intoxicated rats by reducing the lipid peroxide oxidation and preventing mitochondrial enzyme dysfunction. The neutralization of naphthalene-induced dysfunction by the plant extract could be attributed to the antioxidants activity of sulfated polysaccharides in the plant, which is known to quench the free radicals by maintaining mitochondrial enzyme levels (Vijayavel, Anbuselvam & Balasubramanian, 2006).

Recently, the HPLC analyses of condensed tannins from *Rhizophora apiculata* mangrove, followed by depolymerisation in phloroglucinol and ethanol, have successfully identified four terminal units, namely catechin, epicatechin, epigallocatechin and epicatechin gallate. A rapid reversed-phase HPLC method developed for the quantification of condensed tannins identified that catechin was the most predominant constituent of *Rhizophora apiculata* mangrove tannins. The mangrove tannins showed substantial antioxidative activities which were comparable to the synthetic standards and other commercial tannins evaluated (Afidah *et al.*, 2008).

1.7 Antioxidants

Antioxidants are a group of substances which, when present at low concentration compared to that of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell & Gutteridge, 1990). The term "oxidizable substrate" includes almost everything found in living cells, including proteins, lipids, carbohydrates, and deoxyribonucleic acid (DNA). Oxidation is a redox chemical